ELSEVIER

Journal of Immunological Methods 268 (2002) 93–106

JIM
Journal of Immunological Methods

www.elsevier.com/locate/jim

Review

Probing T cell membrane organization using dimeric MHC–Ig complexes

Tarek M. Fahmy, Joan G. Bieler, Jonathan P. Schneck *

Department of Pathology, Division of Immunopathology, Johns Hopkins School of Medicine, 664G Ross Bldg., 720 Rutland Avenue, Baltimore, MD 21205, USA

Received 25 November 2001; accepted 15 April 2002

Abstract

In this report, we review a novel method for probing the membrane organization of T cells using dimeric major histocompatibility complexes (MHC), MHC–Ig. MHC–Ig complexes are useful reagents for quantitative analysis of binding data since their valency is controlled. These complexes can be easily labeled and loaded with a variety of peptides. A binding assay using these dimers and quantitative analysis of the MHC–Ig dimer-T cell binding curves is described in detail. Using this approach, we show that the organization of TCR on activated T cells is different from TCR organization on naïve T cells. The implications of these findings are discussed with regards to current models of T cell recognition. This analysis offers insight into how T cell controls their biological range of responsiveness. Specifically, these findings reveal the biophysical basis of the ability of activated T cells to recognize low amounts of antigen independent of costimulation.

Keywords: T cells; Dimeric MHC; MHC–Ig; TCR organization; Binding assay; Lipid rafts; Fusion constructs; Crosslinks; Clusters; Sensitivity

1. Introduction

Despite recent advances in our understanding of the signal transduction apparatus of T cells, little attention has been focused on how membrane organization of T cells facilitates antigen recognition and the biological responses of T cells. The organization of cell surface molecules, T cell receptors, accessory molecules, and adhesion molecules are crucial factors in facilitating biological responses. The description of the immunological synapse/supramolecular activation clusters (SMACS) (Monks et al., 1998; Grakoui et al., 1999) has significantly contributed to our understanding of the molecular reorganization that accompanies T cell interaction with antigen presenting cells (APC). However, the biophysical variables governing this process are not completely understood. Recently, it has been proposed that cell surface segregation of the TCR on a much smaller scale than the immunologic synapse/SMAC could drive the formation of larger clusters by an active transport process (Van der merwe et al., 2000). To analyze this hypothesis, a method is needed to quantitatively probe TCR organization both on a large scale, as seen with the synapse/SMAC, as well as on a small scale. This analysis will be key in

* Corresponding author. Tel.: +1-410-614-4589; fax: +1-410-614-3589.

E-mail address: Jschnec1@jhmi.edu (J.P. Schneck).

0022-1759/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0022-1759(02)00203-X

accurately correlating TCR organization with physiological responses. Here, we present a novel method for probing T cell membrane organization using dimeric MHC ligands, MHC–Ig complexes. These constructs represent an idealized dimeric antigen that can be used to analyze TCR organization and model T cell–APC interactions.

2. Antigen recognition: puzzles and models

Several barriers have to be overcome for T cells to recognize cognate peptide/MHC ligands effectively on the APC (for a recent review, see Van der Merwe et al., 2000). First, given the large number of peptides presented by the MHC (Falk et al., 1991), a foreign antigen expressed at low density that is likely to be randomly distributed on the cell surface must be reliably recognized. Second, because of the relatively small size of the TCR, 7 nm, compared to other cell surface glycoproteins such as CD45 and CD43, 40–50 nm, the TCR is sterically hindered from contacting its ligand on the APC (Cyster et al., 1991; Shaw and Dustin, 1997). Third, a modest affinity interaction between the MHC–TCR must be translated into a prolonged signal necessary for activation. Finally, saturating concentrations of antibodies specific for the TCR complex are less effective than lower concentrations at triggering T cell responses (Monks et al., 1997).

These barriers for effective recognition of antigen/MHC complexes by the TCR are amplified in the setting of activated T cells, which recognize very low doses of antigen. In one study, a single peptide MHC complex was sufficient to trigger a $CD8^+$ CTL clone (Sykulev et al., 1996). In contrast, naïve T cells require high doses of antigen for a response (Sandberg et al., 1999; Kim et al., 1996). Considering the differences between naïve and activated cells, is there a biophysical mechanism by which T cells are able to modulate responses based on their physiologic state?

The ability of T cells, whether activated or naïve, to recognize antigen despite the barriers for engagement has prompted many studies and models for the process of T cell engagement leading to signaling. A complete model must fulfill three criteria. First, the model must explain how the signal is achieved. Second, the model should explain how the signal is transmitted. Finally, it must predict how the signal is perturbed upon changes in the trigger mechanism. Below, we review four models of T cell triggering that are currently under active investigation.

2.1. Kinetic proofreading models

Some models propose that the key parameter governing T cell activation is the signal duration of the specific peptide/MHC–TCR interaction, which would allow for sufficient time to accumulate proteins required for signal transduction. These models are known as the kinetic proofreading (McKeithan, 1995) or kinetic discrimination (Rabinowitz et al., 1996a) models and were first proposed to suggest a mechanism by which peptide agonists or antagonists result in different T cell activation profiles. Thus, low affinity peptide/MHC complexes stimulate a subset of early activation events such as CD3-zeta chain phosphorylation and calcium release, whereas higher affinity ligands, which take longer to dissociate from the TCR, also stimulate later events such as IL-2 release and target cell lysis (Rabinowitz et al., 1996b; Lyons et al., 1996). The characteristic feature of these models is that signal duration enables the T cell to discriminate between weak and strong antigenic complexes. However, there have been studies, which question the correlation between signal duration and the state of T cell activation (Alam et al., 1996; Kersh et al., 1998; Sykulev et al., 1998). Indeed, it has been shown that a low-affinity TCR ligand with a fast off-rate can stimulate late T cell responses but is unable to stimulate early activation events (Rosette et al., 2001). This has prompted a revision of the kinetic proofreading model to include a cumulative integrated signal, which ‘‘trickles through’’ to the T cell and governs its response. This cumulative signal might for example count the number of effective peptide/MHC engagements and thus, determine the appropriate activation response based on this count (Rosette et al., 2001). However, recently, it has been demonstrated that longer signal durations do not necessarily result in better activation (Kalergis et al., 2001). T cell activation occurs within an optimal duration time window and longer duration times can be detrimental. In addition, these models do not address how the T cell overcomes the initial contact barriers, as summarized above.

2.2. Serial triggering model

Another way to affect an activation event is by increasing TCR occupancy. This could be accomplished with a few peptide/MHC complexes by serial triggering of the TCR (Valitutti et al., 1995). Serial triggering proposes that a single peptide/MHC complex can engage and trigger multiple TCR; thus, achieving a high TCR occupancy and enabling the sensitive detection of a small number of antigenic complexes (Valitutti et al., 1995). This was arrived at by comparing the number of internalized T cell receptors after stimulation with various doses of peptide/MHC complexes assuming that TCR internalization correlated with T cell activation. However, it has been observed that engagement of the peptide/MHC complex can lead to the internalization of non-engaged receptors (San Jose et al., 2000). This indicates that the number of internalized TCR may not correlate with the number of engaged receptors. Thus, it is unclear whether serial triggering also has a component of ligand-independent TCR internalization.

Serial triggering of the TCR is, however, an appealing model since it explains why a low to moderate peptide/MHC complex–TCR affinity is necessary for successful activation. Low to moderate affinity ligands would result in maximizing TCR occupancy, whereas higher affinity ligands would compromise this efficiency by firmly interacting with the TCR complex and dissociating at a slower rate. A recent study however challenged the idea that low to moderate affinity ligands optimally stimulated T cells by showing that a TCR with a significantly higher affinity (300-fold) works efficiently (Holler et al., 2001). Another appealing feature of the serial triggering model is its ability to explain how altered peptide ligands produce differentials in TCR signaling events through differentials in occupancy rates. This model does not however, address how the initial physical barrier to recognition of peptide/MHC complex is overcome.

2.3. Conformational models

It has been proposed that upon encounter with a peptide/MHC complex, the TCR can change to a conformation that induces a signal within the T cell facilitating activation. With the exception of functional data (Rojo and Janeway, 1988) there has been no biophysical or biochemical evidence of TCR conformational changes. To date, TCR crystal structure shows no evidence for gross conformational changes of the TCR following binding to peptide/MHC–TCR complex (Garcia et al., 1999; Ding et al., 1998).

2.4. Clustering and segregation models

Clustering models suggest that upon the initial encounter of the APC with T cell, the TCR dimerizes (Bachmann and Ohashi, 1999) or forms higher order oligomers (Bachmann et al., 1998; Reich et al., 1997) as a prerequisite for T cell activation. Indeed, antibodies that crosslink the CD3 complex or multivalent peptide/MHC complexes can initiate T cell activation (Boniface et al., 1998).

Oligomerization has been observed experimentally in vitro (Alam et al., 1999; Reich et al., 1997). Oligomerization, which was not studied in the setting of the intact cellular plasma membrane, depended on concentration and temperature and whether the ligand was a peptide agonist or antagonist. However, recent studies (Baker and Wiley, 2001) failed to reproduce the SPR-based dimerization or light scattering based oligomerization; casting doubts on the generality of the findings. These authors conclude that oligomerization should be studied in the setting of membrane anchored TCR complexes, as can be done using MHC–Ig.

Since it is difficult to envision how low densities of specific monomeric MHC on the surface of the APC can initiate TCR aggregation on the T cell. This has prompted a revision of this model to include a dynamic component. On initial contact with the APC larger molecules such as CD45, CD43, and LFA-1 segregate to the periphery of the initial contact zone giving way to a smaller cluster of TCR that form opposite the antigenic complex on the APC. This model has been termed the dynamic clustering model (Germain, 1997) or the kinetic segregation model (Van der merwe et al., 2000) and is appealing for three reasons. First, dynamic segregation and clustering of the TCR could contribute to effective signaling by increasing the local concentration of TCR in the contact zone with the APC; thus, increasing the sensitivity of the T cell to low numbers of specific

antigenic complexes. This mechanism, for example, is used by chemotactic bacteria such as *Escherichia coli* to detect low concentrations of attractants (Bray et al., 1998) and provides a sensitive control mechanism that can amplify a weak binding event by a factor of 10^{20} or more (Delisi, 1981). One principle responsible for this mechanism involves an enhanced reaction probability between the ligand and receptor cluster. The larger the receptor cluster, the more likely for a specific ligand to bind longer and hence, the more sensitive the cell becomes to ligand engagement even if it exists at low density. Secondly, clustering of the TCR/CD3 complex concentrates cytoplasmic signaling domains present in CD3, which facilitates efficient signaling. Finally, clustering of the TCR would exclude membrane bound phosphatases such as CD45 from the central cluster and increase peripheral adhesive interactions through the interaction of LFA-1 on the T cell, with ICAM, on the APC, stabilizing the contact zone.

It is important to note that clustering/segregation models do not argue against kinetic proofreading or serial triggering as features of TCR triggering but incorporate them as mechanistic events. Antigenic complexes will dissociate slower from a cluster of TCR than from monovalent segregated receptors hence prolonged signal duration is tied to cluster formation. Serial engagement of TCR is also efficiently facilitated if TCR are preformed in a cluster.

To understand and directly quantitate TCR clustering and the impact of T cell activation on TCR organization and clustering, we used dimeric MHC ligands, MHC–Ig, to probe the T cell membrane. If the clustering/segregation models are accurate then one might expect that TCR clustering is physiologically regulated. Thus, activated T cell, which can recognize low-density antigen in the absence of costimulation may more efficiently cluster TCR than naïve T cells, which require costimulation and high amounts of antigen for efficient signaling.

3. Theory

Binding of MHC–Ig (Fig. 1), which can interact with two TCR simultaneously, on the T cell surface, reflects the avidity of the MHC–Ig ligand for T cells rather than simple MHC–TCR affinity. MHC–Ig complex can be used to distinguish between TCR that are more clustered resulting in higher avidity interactions with the MHC–Ig and TCR that are randomly distributed outside the scanning range of the dimer, resulting in lower avidity binding. As discussed, TCR dimerization has been implicated in T cell activation (Bachmann and Ohashi, 1999); furthermore, the functional signaling unit of the CD3 complex has been shown to consist of two TCR within the same CD3 complex (Exley et al., 1995; Fernandez-Miguel et al., 1999). Therefore, avidity changes may correlate with the physiological state of the T cell.

The avidity of MHC–Ig for T cells is a measure of both the intrinsic affinity of the MHC–TCR interaction (K_d) along with an additional parameter that reports on the efficiency of access to a second receptor nearby (K_x) and the number of receptors available to interact (R_t) (Fig. 2A). This second variable (K_x), which reflects the ability of singly bound ligand to bind a second receptor, is called a cross-linking coefficient or dimerization potential and is exclusive to multivalent ligand-receptor interactions. The product of the dimerization potential and the number of receptors is a dimensionless equilibrium cross-linking constant (K_xR_t). K_xR_t reflects when a monovalently bound dimer (RL) binds through its second site to form a divalently bound complex (R_2L); it is therefore called an enhancement factor (Hornick and Karush, 1972). K_xR_t, the dimensionless cross-linking constant combines two competing factors on the cell surface, the cross-linking potential of receptors and the number of receptors available to fulfill this potential. T cells can have a high cross-linking potential due to enhanced membrane mobility or enhanced TCR preclustering. The total number of receptors independently influences the cross-linking potential. To account for this effect the cross-linking constant (K_x) is factored in with the number of receptors (R_t) into the dimensionless equilibrium cross-linking constant (K_xR_t). Thus, if one can measure this dimerization potential, one can directly and quantitatively report on the state of organization of the receptors on the cell surface.

An overall estimate of the affinity of the dimer for the monovalent receptor cannot be derived (Sawyer and Windsor, 1976). This is apparent from the characteristic concave-upward behavior of Scatchard plots, which describe the binding of multivalent complexes to monovalent receptors (Fig. 2B). Typically

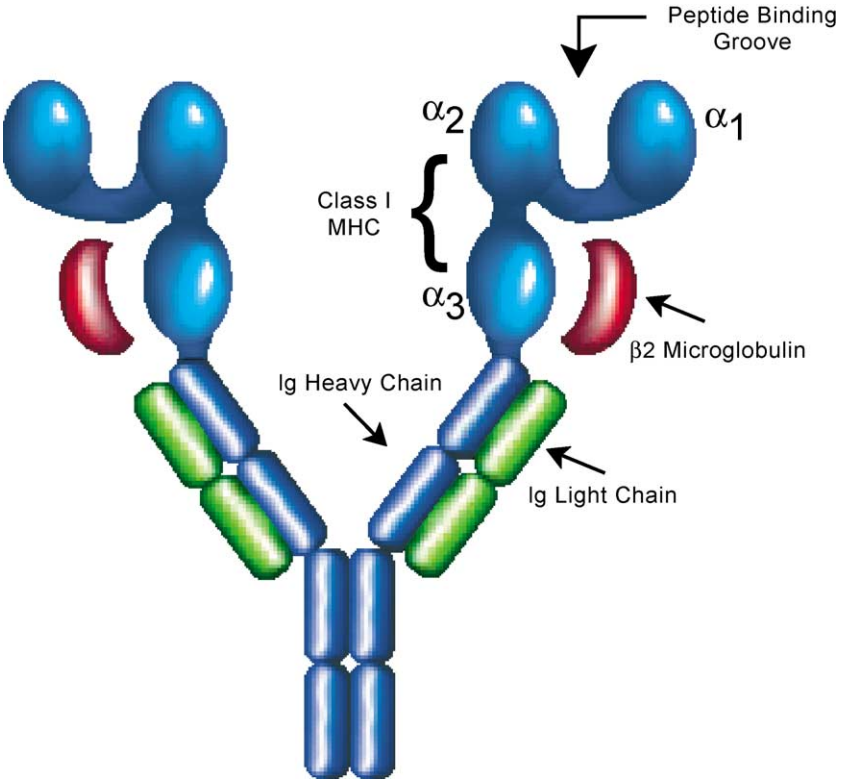

Fig. 1. Schematic of a dimeric MHC, MHC–Ig. A dimeric MHC is constructed by genetically fusing the heavy chain of an antibody to the α_3 domain of a class I MHC. Reprinted with permission (D. Grantham, BDIS).

this means that the effective binding affinity (avidity) decreases as the ligand concentration increases. Thus, no single parameter can describe the overall binding affinity over the entire dimer concentration range. Instead, an approximate affinity can be determined at low and high concentrations (Delisi and Chabay, 1979). The binding avidity, K_v, is related to the fundamental constants of the model described at both low and high dimer concentrations as follows (Lauffenburger and Linderman, 1993):

$$L \rightarrow 0,\ K_v \rightarrow \frac{K_d}{8K_xR_t}$$

$$L \rightarrow \infty,\ K_v \rightarrow \frac{K_d}{2}$$

At low concentrations of the dimer, there is an enhancement to the single-site affinity due to the binding of both subunits. This is reflected in the ratio K_d/K_xR_t. In cases where there is minimal cross-linking potential, K_xR_t is close to one and there is little difference between K_d and K_v. Where the cross linking potential, K_x is high, there is an enhancement of binding due to dimerization, resulting in a measured avidity stronger than the intrinsic single site affinity. However, at high dimer concentrations, dimeric ligands such as MHC–Ig, bind to receptors such as TCR, through just one arm, since an excess of MHC complexes compete for available TCR. Thus, at high concentrations of ligand there is little difference between the single-site affinity constant and the avidity. The statistical factor of two accounts for the fact that dimers can bind by either subunit to monovalent receptors.

In the current paper, we review how the binding avidity of TCR for ligands is physiologically regulated. Upon T cell activation, MHC–Ig have enhanced avidity for TCR. These measurements show that T cells regulate their avidity for multimeric antigen.

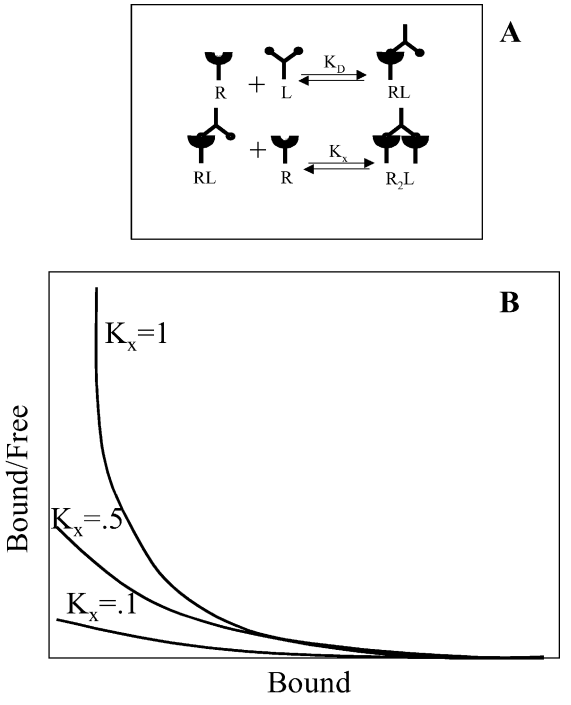

Fig. 2. Equilibrium dimerization of monovalent receptors model. (A) Reaction scheme. A dimeric ligand (L) initially binds to a monovalent receptor to yield a singly bound dimer (RL). The equilibrium coefficient describing this interaction is the intrinsic affinity of the receptor for the ligand (K_d). The singly bound dimer can bind another receptor nearby to form a doubly bound dimer (R_2L). The extent of this interaction is described by the equilibrium crosslinking coefficient (K_x). (B) Scatchard plot representation of reaction as shown in A as a function of the magnitude of the equilibrium crosslinking coefficients (K_x). As the magnitude of this coefficient increases the curvilinearty of the Scatchard plot increases.

The findings strongly support the clustering/segregation model for T cell activation.

4. Methods

4.1. Construction of MHC–Ig

Soluble MHC–Ig fusion proteins were generated by fusing the extracellular domain of a class I MHC to the amino termini of the immunoglobulin heavy chain (IgG1) (Dal Porto et al., 1993; Schneck et al., 2000). These fusion proteins were constructed using pXIg a plasmid containing the cDNA encoding the extracellular region of the MHC and the variable heavy chain of an immunoglobulin gene. These plasmids were then expressed in a murine plasmocytoma cell line, J558L. The result was a dimeric MHC–Ig complex with the heavy immunoglobulin chain (Ig) as a molecular scaffold for the MHC (Fig. 1).

4.2. N-termini labeling of MHC–Ig

Fusion constructs were fluorescently labeled at pH 7.4 with fluorescein isothiocyanate (FITC) (Molecular probes). At this pH, the label is specifically targeted to the amino termini, thus minimizing excessive labeling of lysines present at the receptor-binding interface of the MHC. The fluorescein to protein ratio was typically in the range 1–2 (Fig. 3A). Labeling in this manner did not cause interference in the binding of the MHC–Ig–FITC to TCR as evident from Biacore® binding sensograms of the conjugated and unconjugated MHC–Ig to immobilized TCR (Fig. 3B).

4.3. Peptide loading of MHC–Ig

Another attractive feature of MHC–Ig dimers is the ease of peptide loading. Two different protocols have been developed to efficiently load MHC–Ig complexes with peptides (Schneck et al., 2000). Generally, these protocols are aimed at mildly denaturing the complex followed by refolding in the presence of excess peptide and in some cases with excess β2-microglobulin, which has been found to stabilize the folded conformation of the MHC (Hansen et al., 1988; Rock et al., 1990; Vitiello et al., 1990).

The first peptide loading protocol is an alkaline denaturation and renaturation protocol for efficient loading of K^b-Ig complexes. The second is an acid denaturation and renaturation protocol specific for loading L^d-Ig constructs. In both cases following the addition of a 50-fold molar excess of peptide the solution was neutralized to pH 7.0, and the constructs were allowed to refold for 24–48 h at 4 °C. The amount of peptide loaded was determined using two independent assays: an SPR assay and a fluorescent peptide assay. With both assays we find that 80–90% or greater of MHC–Ig are loaded with specific peptides (data not shown). To ensure that no aggregates formed during the peptide-loading step, the

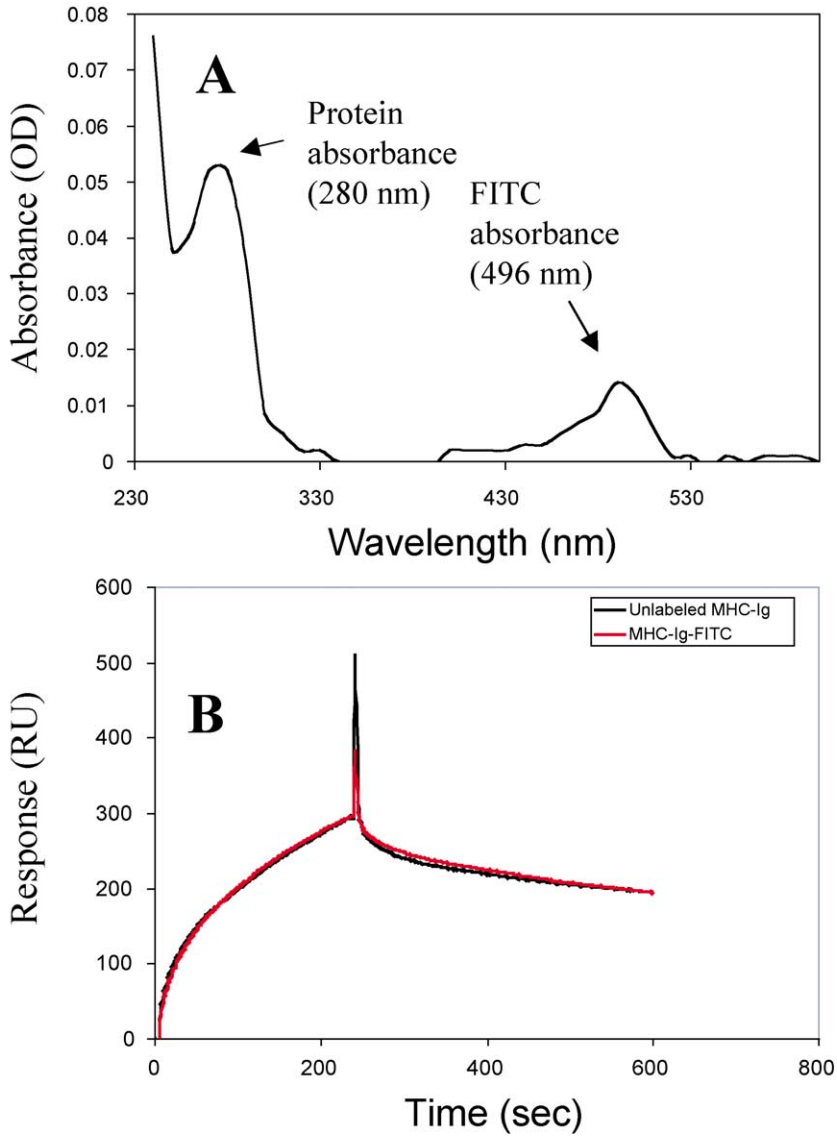

Fig. 3. Preparation and functional assessment of MHC–Ig–FITC complexes. (A) Absorbance profile of H-2L^{d}-Ig–FITC. To avoid excessive labeling, conjugation was performed at pH 7.4 yielding a Fluorescein to protein ratio in the range 1–2. (B) Conjugation of H-2L^{d}-Ig with FITC did not effect its binding to 2C TCR. Conjugated and unconjugated complexes bound similarly to a 2C TCR coated Biacore chip.

complexes were analyzed on a size exclusion column and the amount of protein was quantitated.

4.4. Activated and naïve T cell

The model system studied was the murine class I restricted CD8^{+} 2C T cell response. 2C is an alloreactive, peptide specific, cytotoxic T cell lymphocyte (CTL). This CTL was chosen for two reasons. First, in comparison with other TCR that have been reported, it has high affinities for cognate peptide/MHC ligands (Sykulev et al., 1994; Corr et al., 1994). Second, its peptide/MHC ligands have been well characterized (Sykulev et al., 1994; Al-Ramadi et

al., 1995). It recognizes the peptide, SIYRYYGL (SIY), presented by the syngeneic murine class I H-2K^b MHC, and the peptide, QLSPFPFDL (QL9), presented by the allogeneic murine class I H–2L^d MHC (Sykulev et al., 1994; Udaka et al., 1996).

Naïve T cells were isolated from 2C mouse splenocytes in a sterile environment. These CD8$^+$ cells were enriched to >95% purity using a CD8$^+$ T cell subset enrichment column (R&D Systems MCD8C-1000). A portion of the naïve 2C T cell were activated in vitro with irradiated allogeneic splenocytes (3000 rads) from Balb/C mice (Jackson Labs) in RPMI media supplemented with 10% fetal calf serum (Hyclone).

4.5. MHC–Ig–T cell direct binding assay

Binding of peptide loaded, fluorescently labeled MHC–Ig dimers was measured by flow cytometry using a FACScalibur or a FACSscan (Becton Dickinson). Using this approach, we were able to directly discriminate the amount of MHC–Ig bound to T cells versus that free in solution. Histograms of cell fluorescence (cell number versus fluorescence) in the presence of different concentrations of a specific fluorescent MHC–Ig are compared to the histograms of a non-specific fluorescent ligand. The mean channel fluorescence (MCF) of the liganded T cells increases as the concentration of the bound MHC–Ig ligand increases. To maintain an accurate measure of the concentration of dimer in solution and to minimize cell MCF distortions, all experiments were performed with no washing steps. Specific binding of the dimer was calculated by subtracting non-specific binding from the total binding. These values were then normalized to the maximum specific mean channel fluorescence obtained at saturation. Non-specific binding was determined using K^b-Ig loaded with a K^b-specific peptide derived from ovalbumin-SIINFEKL. Specific binding was determined using the peptide SIY, (SIYRYYGL). For L^d-Ig non-specific binding was determined using the L^d-restricted peptide derived from MCMV (YPHMNTL) and specific binding using the peptide QL9, (QLSPFPFDL). Because the F/P ratio of the dimer complexes was usually low, typical windows for specific binding using this assay were between 20 and 60 MCF.

4.6. Data modeling

Binding data were fit to the equilibrium solution to the dimerization reaction (Perelson, 1984):

$$\mathrm{RL} = R_t\beta\left[\frac{-1+\sqrt{(-1+4\delta)}}{2\delta}\right]$$

$$\mathrm{R_2L} = R_t\left[\frac{1+2\delta-\sqrt{1+4\delta}}{4\delta}\right]$$

$$\beta = \left[\frac{2\mathrm{L}}{K_d+2\mathrm{L}}\right] \quad \text{and} \quad \delta = \beta(1-\beta)K_xR_t$$

The total concentration of bound ligand is $[L_b]=[RL]+[R_2L]$ and the fraction of ligand bound is $[L_b]/R_t$. Three parameters are unknown in these equations, K_d, K_x, and R_t. To determine those parameters, fits of the binding data were performed using the non-linear fitting algorithm of Microcal® Origin 4.1. The resulting three parameters, K_d, K_x, R_t were used to approximate the avidity constant at low concentration, $K_v \sim K_d/K_xR_t$ and to calculate the concentration of singly bound ligand [RL] and concentration of cross-links [R_2L]. The effect of K_d, K_x, R_t on a simulated dimer binding experiment can be observed by downloading the excel simulation file at www.patholgy2/jhu.edu/Schnecklab/.

5. Results

5.1. Membrane organization of activated versus naïve T cells

As previously reported (Fahmy et al., 2001), MHC–Ig dimers bind with enhanced avidity on activated T cells when compared to naïve T cells (Fig. 4A,C). The enhanced avidity is best seen at low ligand concentrations (Fig. 4A,C) and is likely due to increased cross-linking of TCR by the MHC-dimer complexes. This increased cross-linking leads to an increased avidity for dimeric MHC–Ig complex reflected in the marked curvilinearity in the Scatchard plot representation (Fig. 4B,D).

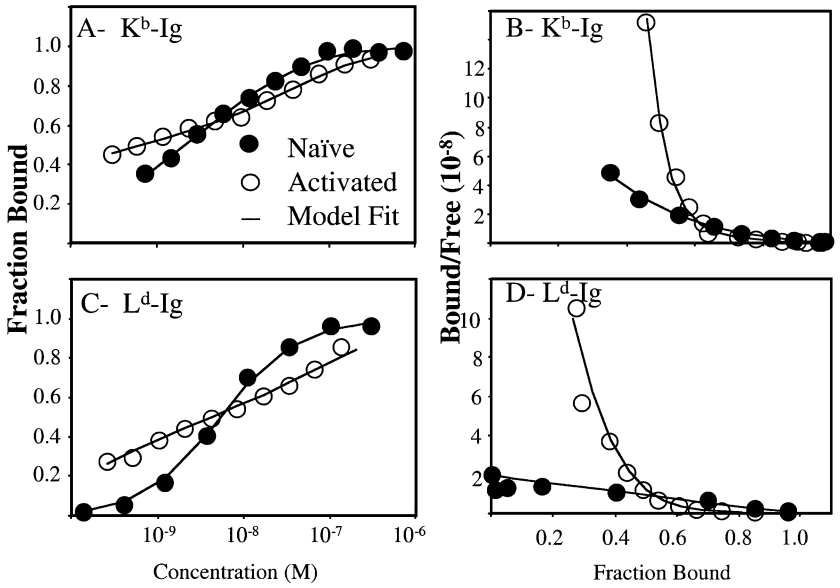

Fig. 4. $^{SIY}K^{b}$-Ig and $^{QL9}L^{d}$-Ig bind naïve and activated T cells with different concentration dependence. MHC dimers were purified and used in flow cytometry as described in Methods. Labeled MHC–Ig dimers were incubated with T cell for 2 h at 4°. Mean channel fluorescence (MCF) value was a measure of the amount of bound ligand. Non-specific binding has been subtracted as described (Fahmy et al., 2001). Only specific binding is shown. Lines through the data points are a non-linear least squares fit using a dimeric ligand-monovalent receptor model (Perelson, 1984). (A, B) Binding isotherms. (C, D) Scatchard representations of the binding isotherms. Reprinted with permission.

When the binding data was deconvoluted, we found that the relationship between the number of crosslinks and the concentration of dimeric ligands was described by a bell-shaped curve (Fig. 5). Interestingly, this bell-shaped curve is observed experimentally for the physiological response of B cells and T cell as a function of antigen dose (Sulzer and Perelson, 1997). The bell shape of the curve arises because the fraction of receptors crosslinked by the dimeric ligand is initially low. As the concentration of dimeric ligand increases the crosslink fraction increases, reaching a maximum. As the concentration continues to increase, crosslinks decrease due to competition between the dimeric ligands for available sites.

5.2. Role of rafts and cytoskeleton in controlling enhanced MHC–Ig binding

One of the major consequences of cellular activation is the compartmentation of TCR and signaling molecules in operationally defined membrane lipid rafts (Janes et al., 2000; Montixi et al., 1998; Simons and Ikonen, 1997; van der Merwe et al., 2000; Viola et al., 1999; Xavier et al., 1998). These lipid rafts are commonly isolated as glycosphingolipid and cholesterol-rich fractions of membrane extracts (Brown and London, 2000). Rafts are dispersed when membrane cholesterol or sphingomylein levels are reduced (Kabouridis et al., 2000). A physiologically regulated change in lipid rafts may contribute to enhanced MHC–Ig binding seen in activated T cells.

Actin also plays an important role in the activation of T cells by APC (Rozdzial et al., 1995; Valitutti et al., 1995). Upon activation, T cells undergo polarization towards the APC where actin filaments have been shown to accumulate at the contact site (Monks et al., 1998; Grakoui et al., 1999). The cytoskeleton is therefore, also likely to be involved in controlling MHC–Ig binding.

To investigate the mechanism leading to increased avidity of TCR on activated T cells, membrane organization was disrupted in four ways. First, we used H57-Fab fragments to physically distance adjoining TCR. H57 recognizes an epitope on the Cβ of the TCR distinct from the TCR–MHC binding interface (Wang et al., 1998). H57-Fabs can therefore, be regarded as a

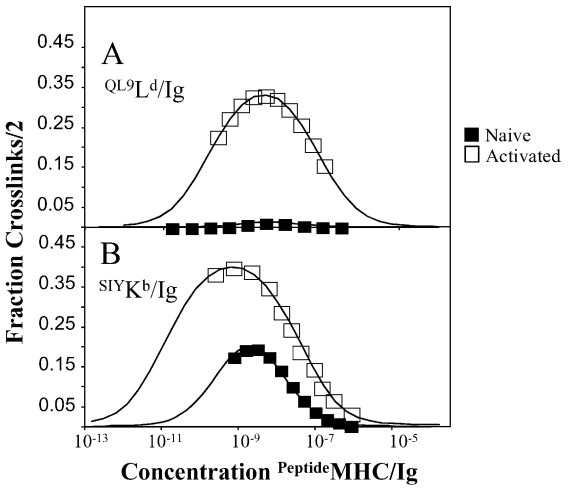

Fig. 5. Concentration of dimer bound by both arms (R_2L), is higher on activated than on naïve T cells. The concentration of crosslinks was obtained by deconvolution of the binding data into two components: RL and R_2L. Deconvolution was performed by substituting the fitted parameters into the single and double bound species equations (see Methods). Points on those curves refer to the experimental range over which the binding experiment was performed. R_2L is shown as a function of $^{QL9}L^d$-Ig (A) and $^{SIY}K^b$-Ig (B) concentration.

"molecular fenders" that can disrupt TCR organization (Fahmy et al., 2001). Second, we chemically depleted membrane cholesterol using Methyl-β-cylcodextrin. Third, membrane rafts were disrupted by depleting membrane sphingomylein using sphingomyleinase. Finally, Latrunculin B, which inhibits F-actin polymerization, was used to investigate the role of the cytoskeleton in the enhanced MHC–Ig binding.

Each method of disruption caused the enhanced avidity of the activated T cell for MHC–Ig dimer to be dramatically lowered (Fig. 6A–D). The curvilinearity seen in Scatchard plots, characteristic of $^{SIY}K^b$-Ig binding to activated cells, was significantly reduced. MHC–Ig dimer binding to H57-Fab-, MβCD-, Sphingomyleinase- and Latrunculin B-treated activated T cell appeared to be similar to dimer binding to naïve T cells. Thus, enhanced MHC–Ig binding to activated T cells could be disrupted by use of "molecular fenders" or by disrupting raft organization or the cytoskeleton.

Reduction in the cross-linking potential following actin disruption suggests that the mechanism responsible for TCR reorganization has been destabilized.

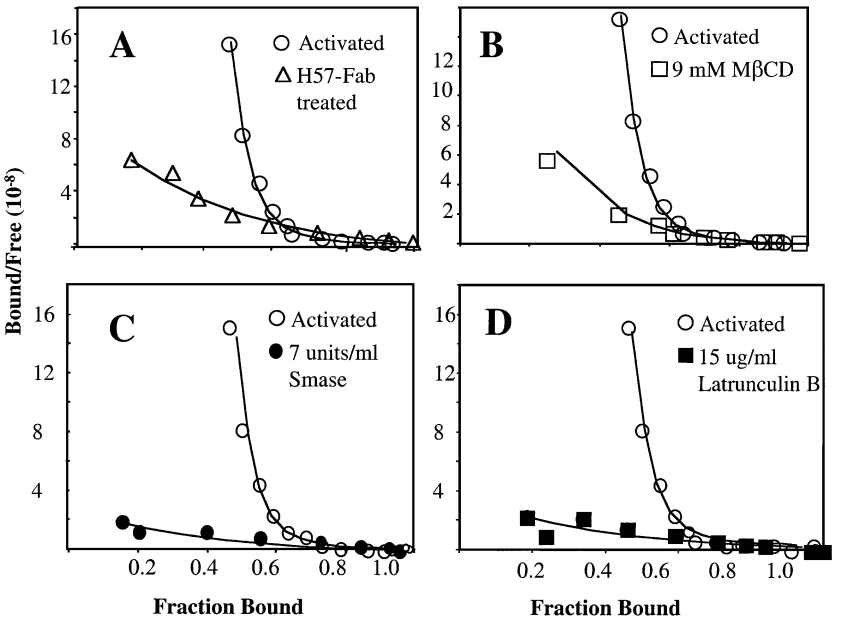

Fig. 6. Avidity of $^{SIY}K^b$–Ig to activated T cells is reduced by treatment with (A) H57-Fabs that sterically segregate receptors; (B) MβCD (9 mM), which extracts membrane cholesterol; (C) Sphingomyleinase (7 units/ml), which depletes membrane sphingomylein; and (D) Latrunculin B (15 μg/ml), which disrupts actin polymerization. Sphingomyleinase, MbCD and Latrunculin B treatments were performed at 37 °C for 15–30 min. H57-Fabs were incubated with T cells at 4 °C for 30 min.

Disruption of raft structures by compromising actin polymerization is consistent with studies showing that the engagement of T cells with APC induces actin recruitment and stabilizes raft domains (Lowin-Kropf et al., 1998). Indeed, it has been suggested that actin polarization in stimulated T cells functions to transport raft domains to the site of TCR engagement (Rodgers and Zavzavadjia, 2001). If this transport mechanism is disrupted then the formation of TCR associated raft domains is halted which results in a decrease of the avidity of dimeric peptide/MHC.

5.3. Kinetics of binding of MHC–Ig to activated and naïve T cells

The kinetics of MHC–Ig association and dissociation is an indication of TCR organization on the membranes of activated and naïve T cells. Reorganization of TCR leading to clustering is expected to yield slower dissociation rates. The magnitude of this decrease is dependent on the cluster size. In contrast, cluster size should have minimal impact on the association rates of MHC–Ig complexes (Delisi, 1980). Therefore, analysis of the kinetics of binding should help us understand the role of TCR cluster formation in activated versus naïve T cells.

The dissociation rate of $^{SIY}K^b$-Ig from activated T cells is longer than the dissociation rate from naïve cells (Fig. 7A). Data were fit to a monophasic dissociation model: $y = y_o + A_1 \exp(-(t - x_o)/t_1)$. The apparent off-rate of the dimer from the surface of activated cells is approximately 4-fold slower than it is from naïve cells.

The slower dissociation rate is likely to be related to enhanced cross-linking due to surface clustering. The intrinsic dissociation rates of dimeric ligands (k_{off}) are more rapid than cross-linking formation (k_x) (Pecht and Lancet, 1977; Berg and Purcell, 1977), i.e., a dimeric ligand will be bind and dissociate many times before successfully cross-linking two receptors. Thus, the observed differences in dissociation rates between naïve and activated T cells reflect the differences in the dissociation of crosslinks since the intrinsic dissociation rates are taking place on a faster time scale; beyond the resolution limits of this assay.

The apparent off-rate of MHC–Ig is slowed down proportionally by the size of the cluster and may be quantitated using: $K_{off} = 2.5D/d^2$ (Delisi, 1980) where

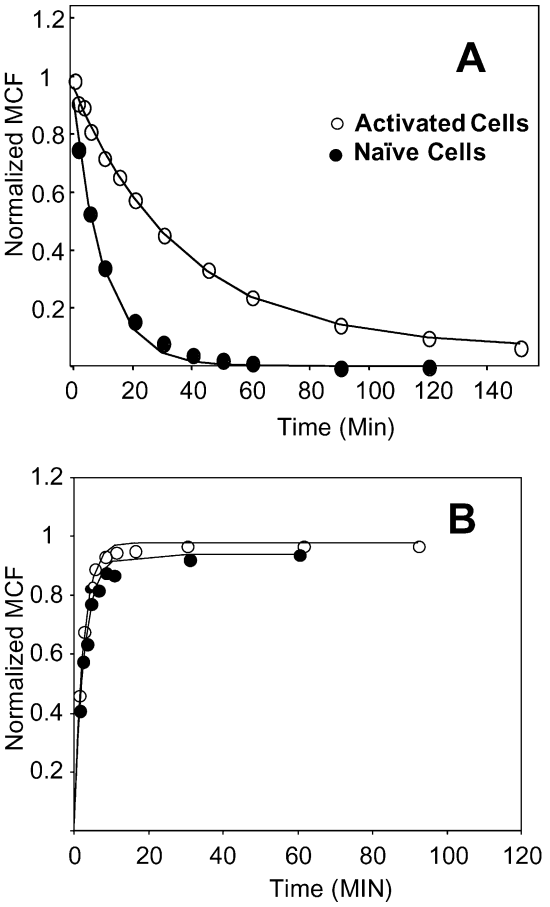

Fig. 7. (A) Kinetics of dissociation of 200 nM $^{SIY}K^b$-Ig are slower on activated T cells versus naïve T cells. MHC–Ig dissociation kinetics were measured by competition with a 50-fold excess unlabeled antibody specific for 2C TCR (1B2 antibody). (B) Association kinetics of $^{SIY}K^b$-Ig are unchanged. Association was measured by incubation of specific $^{SIY}K^b$-Ig and non-specific $^{SIIN}K^b$-Ig dimeric MHC at 4 °C with T cells. At the times shown, the MCF of an aliquot was read. The amount of specific binding at the indicated times was determined by subtraction of the non-specific $^{SIIN}K^b$-Ig MCF from the total $^{SIY}K^b$-Ig MCF.

D is the effective diffusion constant of receptors within the T cell membrane and d is the size of the cluster. Diffusion coefficients on cell membranes are typically in the range 10^{-10} cm^2/s (Dragsten et al., 1979). For the apparent off-rates predicted by (Fig. 7A), we derive a 2-fold increase in the size of the cluster on activated versus naive cells, which is consistent with a 2-fold increase in the equilibrium number of crosslinks (Fig. 5).

Cluster size also indirectly influences the off-rate through its dependence on the translational diffusion

coefficient of the receptors (D). Receptor clusters will diffuse less and decreased mobility will contribute to a slower apparent dimer off-rate.

In contrast to the dissociation rates, the observed association rates remain unchanged as measured by this assay (Fig. 7B). Here data were fit to the monophasic association model: $y = y_o + A_2(1 - \exp(-(k_{on}C + k_{off})t)$, where k_{on} is the association rate and k_{off} is the dissociation rate determined from Fig. 7A, and C is the concentration of dimeric ligand. The association rates remain unchanged due to the fact that the forward rate of association of dimer with a cluster is only weakly dependent on the cluster size, in fact, inversely logarithmic (Delisi, 1980). This is quantitatively expressed as follows: $K_{on} \sim D/L_n(x/d)$, where x is the distance between the receptors. Thus, for moderate differences in cluster size as observed for naïve and activated T cells, the model predicts that the forward rate constants for the association of MHC–Ig dimer with the cluster will not change significantly.

6. Conclusion

We have used dimeric MHC–Ig complexes to quantitatively probe the surface of T cells in different physiological states. Activated and naïve T cells bind MHC–Ig with different concentration dependence. Binding data fit a model where the dimeric MHC is able to crosslink more receptors on the surface of activated T cells than on naïve T cells. A dimensionless cross-linking equilibrium constant, K_xR_t, was used to quantitate the enhanced cross-linking. Together with the intrinsic affinity, K_xR_t was used to estimate the overall avidity of MHC–Ig to T cells. This avidity measure reveals an enhancement of the intrinsic affinity on activated as compared to naïve T cells as a result of receptor cross-linking.

Kinetic analysis of MHC–Ig dimer binding also reveals additional interesting findings. While association kinetics of the dimer to both activated and naive T cells does not differ significantly, dissociation kinetics are 4-fold slower on activated T cells consistent with a mechanism of enhanced clustering on the surface of activated versus naïve T cells. Enhanced cross-linking is a consequence of TCR reorganization on activated T cells.

The results also show that activation induced T cell membrane reorganization is facilitated by the formation or aggregation of lipid rafts. Agents that disrupt their proximity, either sterically by the addition of H57 Fabs, or chemically by the extraction of integral components of lipid rafts such as membrane cholesterol or sphingomylein, compromise the integrity of TCR organization on activated T cells. In addition, disruption of the actin cytoskeleton effected TCR reorganization suggesting an active redistribution of TCR and lipids mediated by the cytoskeleton.

In the introduction to this review, we discussed barriers that T cells need to overcome to effectively engage cognate peptide/MHC complexes. Sparse ligand concentrations, low affinities and steric hindrance all work against efficient recognition of peptide/MHC ligands by TCR. Our data show that TCR crosslink more efficiently on activated T cells. This data provides an explanation for the way in which barriers to TCR engagement may be overcome. First, enhanced peptide/MHC–TCR avidities translate into prolonged T cell signaling necessary for T cell activation. Second, TCR rearrangement and clustering may eliminate steric constraints posed by longer membrane molecules that may impede peptide/MHC–TCR interactions. Third, TCR clustering amplifies the response of a single TCR interaction; thus, a low density of antigen is sufficient to affect a T cell response. Finally, this data predicts that very high levels of antigen presentation may lead to a decrease in T cell signaling by decreasing TCR cross-linking.

Acknowledgements

We thank Michael Edidin and Georg Russwurm for valuable comments. Support for this work was provided by grants from the NIH AI-29575, AI-44129, and AI-14584, and in part provided by Pharmingen/BDIS. MHC–Ig are licensed to Pharmingen/BDIS and marketed as DimerX.

References

Alam, S.M., Travers, P.J., Wung, J.L., Nasholds, W., Redpath, S., Jameson, S.C., Gascoigne, N.R., 1996. T cell receptor affinity and thymocyte positive selection. Nature 381, 616–620.

Alam, S.M., Davies, G.M., Lin, C.M., Zal, T., Nasholds, W., Jameson, S.C., Hogquist, K.A., Gascoigne, N.R.J., Travers, P.J., 1999. Qualitative and quantitative differences in T cell receptor binding of agonist and antagonist ligands. Immunity 10, 227–237.

Al-Ramadi, B.K., Jelonek, J.T., Boyd, L.F., Margulies, D.H., Bothwell, A.L., 1995. Lack of strict correlation of functional sensitization with the apparent affinity of MHC/peptide complexes for the TCR. J. Immunol. 155, 662–673.

Bachmann, M.F., Ohashi, P.S., 1999. The role of T-cell receptor dimerization in T cell activation. Immunol. Today 20, 568–576.

Bachmann, M.F., Salzmann, M., Oxenius, A., Ohashi, P., 1998. Formation of TCR dimers/trimers as a crucial step for T cell activation. Eur. J. Immunol. 28, 2571–2579.

Baker, B.M., Wiley, D.C., 2001. Alpha beta T cell receptor ligand-specific oligomerization revisited. Immunity 6, 681–692.

Berg, H.C., Purcell, E.M., 1977. Physics of chemoreception. Biophys. J. 20, 193–219.

Boniface, J.J., Rabinowitz, J.D., Wulfing, C., Hampl, J., Reich, Z., Altman, J.D., Kantor, R.M., Beeson, C., McConnel, H.M., Davis, M.M., 1998. Initiation of signal transduction through the T cell receptor requires the peptide multivalent engagement of MHC ligands. Immunity 9, 459–466.

Bray, D., Levin, M., Morton-Firth, C.J., 1998. Receptor clustering as a cellular mechanism to control sensitivity. Nature 393, 85–88.

Brown, D.A., London, E., 2000. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J. Biol. Chem. 27523, 17221.

Corr, M., Slanetz, A.E., Boyd, L.F., Jelonek, M.T., Khiko, S., Al-Ramadi, B.K., Kim, Y.S., Maher, S.E., Bothwell, A.L., Margulies, D.H., 1994. T cell receptor–MHC Class I peptide interactions: affinity, kinetics and specificity. Science 265, 946–949.

Cyster, J.G., Shotton, D.M., Williams, A.F., 1991. The dimensions of the T lymphocyte glycoprotein leukosialin and identification of linear protein epitopes that can be modified by glycosylation. EMBO J. 19, 893–902.

Dal Porto, J., Johansen, T.E., Catipovic, B., Parfitt, D.J., Tuveson, D., Gether, U., Kozlowski, S., Fearon, D., Schneck, J.P., 1993. A soluble divalent class I major histocompatibility complex molecule inhibits alloreactive T cells at nanomolar concentrations. Proc. Natl. Acad. Sci. U. S. A. 90, 6671–6675.

Delisi, C., 1980. Theory of clustering of cell surface receptors by ligands of arbitrary valence: dependence of dose response patterns on a coarse cluster characteristic. Math. Biosci. 52, 159.

Delisi, C., 1981. The magnitude of signal amplification by ligand-induced receptor clustering. Nature 289, 322–323.

Delisi, C., Chabay, R., 1979. The influence of cell surface receptor clustering on the thermodynamics of ligand binding and the kinetics of its dissociation. Cell Biophys. 1, 117–131.

Ding, Y.H., Smith, K.J., Garboczi, D.N., Utz, U., Biddison, W.E., Wiley, D.C., 1998. Two human T cell receptors bind in a similar diagonal mode to the HLA-A2/Tax peptide complex using different TCR amino acids. Immunity 4, 403–411.

Dragsten, P., Henkart, P., Blumenthal, R., Weinstein, J., Schlessinger, J., 1979. Lateral diffusion of surface immunoglobulin, Thy-1 antigen, and a lipid probe in lymphocyte plasma membranes. Proc. Natl. Acad. Sci. U. S. A. 6, 5163–5167.

Exley, M., Wileman, T., Mueller, B., Terhorst, C., 1995. Evidence for multivalent structure of T-cell antigen receptor complex. Mol. Immunol. 32, 829–839.

Fahmy, T.M., Bieler, J.G., Edidin, M., Schneck, J.P., 2001. Increased TCR avidity after T cell activation: a mechanism for sensing low-density antigen. Immunity 2, 135–143.

Falk, K., Rotzchke, O., Stevanovic, S., Jung, G., Rammensee, H.G., 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 351, 290–296.

Fernandez-Miguel, G., Alarcon, B., Iglesias, A., Bluethmann, H., Alvarez-Mon, M., Sanz, E., de la Hera, A., 1999. Multivalent structure of an alpha–beta T cell receptor. Proc. Natl. Acad. Sci. U. S. A. 96, 1547–1552.

Garcia, K.C., Teyton, L., Wilson, I.A., 1999. Structural basis of T-cell recognition. Annu. Rev. Immunol. 17, 369–397.

Germain, R.N., 1997. T-cell signaling: the importance of receptor clustering. Curr. Biol. 1 (710), R640–R644. Oct., Review.

Grakoui, A., Bromley, S.K., Sumen, C., Davis, M.M., Shaw, A.S., Allen, P.M., Dustin, M.L., 1999. The immunological synapse: a molecular machine controlling T cell activation. Science 285, 221.

Hansen, T.H., Myers, N.B., Lee, D.R., 1988. Studies of two antigenic forms of Ld with disparate β2-microglobulin associations suggest that β2m facilitates the folding of α1 and α2 domains during de novo synthesis. J. Immunol. 140, 3522.

Holler, P.D., Lim, A.R., Cho, B.K., Rund, L.A., Kranz, D.M., 2001. CD8-T cell transfectants that express a high affinity T cell receptor exhibit enhanced peptide-dependent activation. J. Exp. Med. 8, 1043–1052.

Hornick, C.L., Karush, F., 1972. Antibody affinity. 3. Role of Multivalence. Immunochemistry 9, 325–333.

Janes, P.W., Ley, S.C., Magee, A.I., Kabouridis, P.S., 2000. The role of lipid rafts in T cell antigen receptor TCR signalling. Semin. Immunol. 12, 23–34.

Kabouridis, P.S., Janzen, J., Magee, A.L., Ley, S.C., 2000. Cholesterol depletion disrupts lipid rafts and modulates the activity of multiple signaling pathways in T lymphocytes. Eur. J. Immunol. 30, 954–963.

Kalergis, A.M., Boucheron, N., Doucey, M.A., Palmieri, E., Goyarts, E.C., Vegh, Z., Luescher, I.F., Nathenson, S.G., 2001. Efficient T cell activation requires an optimal dwell-time of interaction between the TCR and the pMHC complex. Nat. Immunol. 3, 229–234.

Kersh, G.J., Kersh, E.N., Fremont, D.H., Allen, P.M., 1998. High and low potency ligands with similar affinities for the TCR: the importance of kinetics in TCR signaling. Immunity 9, 817–826.

Kim, D.T., Rothbar, J.B., Bloom, D.D., Fathman, C.G., 1996. Quantitative analysis of T cell activation: role of TCR/ligand density and TCR affinity. J. Immunol. 156, 2737–2742.

Lauffenburger, D.A., Linderman, J.J., 1993. Receptors: Models for Binding, Trafficking, and Signaling. Oxford Univ. Press, pp. 133–180.

Lowin-Kropf, B., Shapiro, V.S., Weiss, A., 1998. Cytoskeletal polarization of T cell is regulated by an immunoreceptor tyrosine based activation motif-dependent mechanism. J. Cell Biol. 140, 861–871.

Lyons, D.S., Lieberman, S.A., Haml, J., Boniface, J.J., Chien, Y., Berg, L.J., Davis, M.M., 1996. A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. Immunity 5, 53–61.

McKeithan, T.W., 1995. Kinetic proofreading in T cell receptor signal transduction. Proc. Natl. Acad. Sci. U. S. A. 93, 1401–1405.

Monks, C.R., Kupfer, H., Tamir, I., Barlow, A., Kupfer, A., 1997. Selective modulation of protein kinase C-theta during T cell activation. Nature 385, 83–86.

Monks, C.R.F., Freiberg, B.A., Kupfer, H., Sciaky, N., Kupfer, A., 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. Nature 395, 82.

Montixi, C., Langlet, C., Bernard, A.M., Thimonier, J., Dubois, C., Wurbel, M.A., Chauvin, J.P., Pierres, M., He, H.T., 1998. Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. EMBO J. 17, 5334–5348.

Pecht, I., Lancet, O., 1977. Kinetics of antibody–hapten interactions. In: Pecht, I., Ringler, R. (Eds.), Chemical Relaxation in Molecular Biology. Springer-Verlag, New York.

Perelson, A.S., 1984. In: Perelson, A.S., Delisi, C., Wiegel, F.W. (Eds.), Cell Surface Dynamics: Concepts and Models. Marcel Dekker, New York, pp. 223–276.

Rabinowitz, J.D., Beeson, C., Lyons, D.S., Davis, M.M., McConnell, H.M., 1996a. Kinetic discrimination in T cell activation. Proc. Natl. Acad. Sci. U. S. A. 93, 1401–1405.

Rabinowitz, J.D., Beeson, C., Wulfing, C., Tate, K., Allen, P.M., Davis, M.M., McConnel, H.M., 1996b. Altered T cell receptor ligands trigger a subset of early T cell activation signals. Immunity 5, 125–135.

Reich, Z., Boniface, J.J., Lyons, D.S., Borochov, N., Wachtel, E.J., Davis, M.M., 1997. Ligand-specific oligomerization of T-cell receptor molecules. Nature 387, 617–620.

Rock, K.L., Rothstein, L.E., Gamble, S.R., Benacerraf, B., 1990. Reassociation with β2-micrOglobulin is necessary for Kb class I major histocompatibility complex binding of exogenous peptides. Proc. Natl. Acad. Sci. U. S. A. 87, 7517.

Rodgers, W., Zavzavadjia, J., 2001. Glycolipid-enriched membrane domains are assembled into membrane patches by associating with the actin cytoskeleton. Exp. Cell Res. 267, 173–183.

Rojo, J.M., Janeway, C.A., 1998. The biologic activity of anti-T cell receptor V region monoclonal antibodies is determined by the epitope recognized. J. Immunol. 140, 1081–1088.

Rosette, C., Werten, G., Daniels, M., Hollman, P., Alam, S., Travers, P., Gascoigne, N., Palmer, E., Jameson, S., 2001. The impact of duration versus extent of TCR occupancy on T cell activation: a revision of the kinetic proofreading model. Immunity 16, 59–70.

Rozdzial, M.M., Malissen, B., Finkel, T.H., 1995. Tyrosine-phosphorylated T cell receptor zeta chain associates with the actin cytoskeleton upon activation of mature T lymphocytes. Immunity 5, 623–633.

San Jose, E., Borroto, A., Niedergang, F., Alcover, A., Alacron, B., 2000. Triggering the TCR complex causes the downregulation of nonegaged receptors by a signal transduction-dependent mechanism. Immunity 12, 161–170.

Sandberg, J.K., Karre, K., Glas, R., 1999. Monitoring the major histocompatibility complex restriction element modulates $CD8^+$ T cell specificity and compensates for loss of T cell receptor contacts with the specific peptide. J. Exp. Med. 189, 883–894.

Sawyer, W.H., Windsor, D.J., 1976. Thermodynamic requirements for the cross-linking theory of lymphocyte activation: the interpretation of dose–response curves. Immunochemistry 13, 141–147.

Schneck, J.P., Slansky, J.E., O'Herrin, S.M., Greten, T.F., 2000. Monitoring antigen specific T-cell using MHC–Ig dimers. Curr. Protoc. Immunol. Suppl. 35, 17.2.1–17.2.17.

Shaw, A.S., Dustin, M.L., 1997. Making the T cell receptor go the distance: a topological view of T cell activation. Immunity 6, 361–369.

Simons, K., Ikonen, E., 1997. Functional rafts in cell membranes. Nature 387, 569–572.

Sulzer, B., Perelson, A.S., 1997. Immunons revisited: binding of multivalent antigens to B-cells. Mol. Immunol. 1, 63–74.

Sykulev, Y., Brunmark, A., Tsomides, T.J., Kageyama, S., Jackson, M., Peterson, P.A., Eisen, H.N., 1994. High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogeneic and syngeneic major histocompatibility complex class I proteins. Proc. Natl. Acad. Sci. U. S. A. 91, 11487–11491.

Sykulev, Y., Joo, M., Vturina, I., Tsomides, T.J., Eisen, H.N., 1996. Evidence that a single peptide–MHC complex on a target cell can elicit a cytolytic T cell response. Immunity 4, 565.

Sykulev, Y., Vugmeyster, Y., Brunmark, A., Ploegh, H.L., Eisen, H.N., 1998. Peptide antagonism and T cell receptor interactions with peptide–MHC complexes. Immunity 9, 475–483.

Udaka, K., Wiesmuller, K.-H., Kienle, S., Jung, G., Walden, P., 1996. Self–MHC–restricted peptides recognized by an alloreactive T lymphocyte clone. J. Immunol. 157, 670–678.

Valitutti, S., Dessing, M., Aktories, K., Gallati, H., Lanzavecchia, A., 1995. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. J. Exp. Med. 181, 577–584.

Van der Merwe, P.A., Davis, S.J., Shaw, A.S., Dustin, M.L., 2000. Cytoskeletal polarization and redistribution of cell surface molecules during T cell antigen recognition. Sem. Immunol. 12, 5–21.

Viola, A., Schroeder, S., Sakakibara, Y., Lanzavecchia, A., 1999. T lymphocytes costimulation mediated by reorganization of membrane microdomains. Science 283, 680–682.

Vitiello, A., Potter, T.A., Sherman, L.A., 1990. The role of β2-microglobulin in peptide binding by class I molecules. Science 250, 1423.

Wang, J., Lim, K., Smolyar, A., Teng, M., Liu, J., Tse, A.G., Liu, J., Hussey, R.E., Chishti, Y., Thomson, C.T., Sweet, R.M., Nathenson, S.G., Chang, H.C., Sacchettini, J.C., Reinherz, E.L., 1998. Atomic structure of an alpha–beta T cell receptor TCR heterodimer in complex with an anti-TCR fab fragment derived from a mitogenic antibody. EMBO J. 17, 10–26.

Xavier, R., Brennan, T., Li, Q., McCormack, C., Seed, B., 1998. Membrane compartmentation is required for efficient T cell activation. Immunity 8, 723–732.